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European Journal of Pharmacology 428 (2001) 261–267

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The role of nitric oxide in the reversal of hemorrhagic shock by oxotremorine

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Received 9 July 2001; received in revised form 8 August 2001; accepted 10 August 2001

Abstract

In the present study, the effect of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME), on the antishock actions of oxotremorine was investigated in rats subjected to hemorrhagic shock under urethane anesthesia. L-citrulline production in the AV3V region, as an indicator of nitric oxide (NO) synthesis, was assayed by high-performance liquid chromatography (HPLC) with fluorescent detection throughout the experiment. The rats were pretreated with either intravenous (i.v.) physiological saline or L-NAME (2.5 mg/kg) before bleeding. L-NAME potentiated the reversal of hypotension by oxotremorine (25 μg/kg, i.v.). However, oxotremorine either alone or in combination with L-NAME did not produce any significant change in 60-min survival rate at this low dose. Analysis of microdialysis samples collected from the AV3V region showed that L-citrulline concentration increased during bleeding and that this increase was abolished by L-NAME pretreatment. These results may suggest that nitric oxide production contributes to hypotension in rats bled to shock since nitric oxide levels in the AV3V region increased in response to bleeding and nitric oxide synthase (NOS) inhibition abolished this increase and potentiated the oxotremorine-induced reversal of hypotension.

Keywords: Anteroventral third ventricle region; Hemorrhagic shock; L-Citrulline; N^G-nitro-L-arginine methylester (L-NAME); Microdialysis; Muscarinic receptor; Nitric oxide (NO); Oxotremorine; (Rat)

1. Introduction

Stimulation of central muscarinic receptors in rats results in hypertension, primarily through an increase in sympathetic outflow to the vasculature (Brezenoff and Giuliano, 1982). The administration of cholinomimetics directly into the cerebral ventricles or into several specific sites such as the posterior hypothalamic nucleus, the ventrolateral medullary pressor area, hippocampus, locus ceruleus and C1 area of the rostral ventrolateral medulla was reported to induce a pressor response (Buccafusco and Brezenoff, 1979; De Luca et al., 1990; Giuliano et al., 1989; Haruta et al., 1992; Martin, 1992; Nattie and Li, 1990; Sundaram et al., 1988). It was also shown that the anteroventral 3rd ventricle (AV3V) region is involved in the cholinergic central regulation of the cardiovascular system through pressor, dipsogenic and natriuretic responses (Menani et al., 1990). Electrolytic lesions of this region prevent the development of salt-induced hypertension (Sanders and Johnson, 1989).

Centrally active cholinomimetic agents have been reported to improve hypotension and increase survival rate in rats subjected to severe hemorrhagic shock (Guarini et al., 1989; Onat et al., 1994). Onat et al. (1994) have reported that muscarinic receptors are involved in the reversal of hypotension whereas nicotinic receptors were responsible for the increase in survival rate. This study also showed that electrolytic lesioning of the AV3V region prevented the pressor effect of oxotremorine in this model (Onat et al., 1994).

On the other hand, adrenocorticotropic hormone (ACTH) produces a sustained reversal of hemorrhagic shock in rats via central cholinergic neurons and muscarinic receptors (Bertolini et al., 1989; Guarini et al., 1990). The same researchers also reported that nitric oxide synthase (NOS) inhibition by N^G-nitro-L-arginine methylester (L-NAME) and *S*-methylisothiourea potentiated the effects of adrenocorticotropic hormone, suggesting an overproduction of nitric oxide (NO) in hemorrhagic shock (Bazzani et al., 1989). Therefore, it may be assumed that NO may also counteract cholinomimetic-induced pressor and/or antishock effects of cholinomimetic agents. NO is a free radical, generated by NOS, utilizing L-arginine as

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01294-8

substrate and L-citrulline is formed as a by-product (Bredt and Snyder, 1994). L-citrulline is considered to be produced exclusively by NOS, since the urea cycle in brain tissue is incomplete and L-citrulline immunocytochemistry was reported to be a convenient means of studying NOS activity (Keilhoff et al., 2000).

The present study was designed to investigate whether the NO synthase inhibitor, L-NAME, would alter oxotremorine-induced effects on blood pressure and short-term survival of rats in hemorrhagic shock under urethane anesthesia. The participation of NO in the AV3V region was investigated by measuring the extracellular concentration of L-citrulline in microdialysis samples.

2. Materials and methods

2.1. Animals

Experiments were performed with albino Sprague Dawley rats of both sexes, weighing 200–250 g. All animals were fed a standard diet with water ad libitum, and kept at room temperature (20 ± 3 °C) in an air-conditioned room with a 12-h light/dark cycle. Experiments were performed under urethane (1.2 g/kg, i.p.) anesthesia. Normal body temperature was maintained by continuous monitoring via a rectal thermometer and a heating pad during the experiments. All procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Direct measurement of blood pressure

Both iliac arteries were catheterized with a PE-10 catheter attached to PE-50 polyethylene tubing, one for direct measurement of blood pressure, and the other for bleeding. Arterial blood pressure was recorded on a polygraph (Grass Model 7, USA) via a pressure transducer (Grass). The left iliac vein was also catheterized for intravenous administration of drug solutions. All catheters were filled with 1% heparin–saline solution.

2.3. Microdialysis: probe placement and procedure

The shaft of concentric microdialysis probes was made of 24 gauge stainless-steel tubing (15 mm) (Cooper's Needle Works, UK) as described previously (Obrenovitch et al., 1995). Inlet and outlet tubes were made of fused silica (outer diameter: 0.19 mm; inner diameter: 0.075 mm) (SGE, UK). The fused silica tubes were inserted into the steel tubing under a surgical microscope and the inlet emerging from the tip of the tubing was trimmed to a length of 2 mm. A cuprophan dialysis membrane (outer diameter: 0.216 mm; inner diameter: 0.2 mm) (Gambro, UK) was inserted over the inlet of the silica tubing. All joints of the probes were sealed with epoxy resin. The dialyzable part of the probe was 2.5 mm in length.

The microdialysis probe was placed into the AV3V region with the coordinates of 0.0–0.3 mm posterior to the bregma at midline and 8.5 mm ventral from the skull surface (Paxinos and Watson, 1986). A supporting screw was placed on the skull and the probe was sealed with dental acrylic cement. For dialysate collection, a polyethylene tube was attached to the inlet of the probe and artificial cerebrospinal fluid (aCSF) was delivered continuously (0.5 μl/min) via a 500-μl Hamilton syringe connected to a microinfusion pump (KDS Scientific, USA). The composition of aCSF was as follows (in mM): KCl, 2.5; NaCl, 125; $CaCl_2$, 1.26, $MgCl_2$, 1.18, NaH_2PO_4, 0.2. The aCSF had a pH set to 7.0 and was filtered through 0.4-μm nylon membrane filters before perfusion. Samples were collected after a 2–3-h equilibration period. The dialysates accumulated in 0.5-ml eppendorf tubes and the tubes were changed every 20 min. Two basal samples, one sample during hemorrhage and three consecutive samples after oxotremorine injection were collected. The dialysates were stored at −20 °C and assayed the following day.

2.4. Histological verification of microdialysis probes

After completion of the experiments, the rats were decapitated and the brains were put in 4% sucrose solution in 20% formol. Forty-micrometer coronal sections were taken with a cryostat at −20 °C (Microm, Germany). The slices were then stained with thionine and checked for probe placement. Improper placements were not included in the data analysis. The coronal brain section and the exact location of the probe placement are shown schematically in Fig. 1. Proper probe placement was defined as below the level of the anterior commissure in the periventricular tissue between the preoptic, anterior hypothalamic periventricular and median preoptic nuclei.

2.5. L-Citrulline assay

L-Citrulline levels in the dialysates obtained from AV3V regions of rats subjected to hemorrhagic shock were measured via high-performance liquid chromatography (HPLC) as modified from Biggs et al. (1992). The chromatographic system consisted of a pump (Jasco PU-980, Japan) with a 100-μl loop and a rheodyne valve, C18 reverse phase nucleosil working and guard columns (15 and 2.5 cm in length, 4.6 μm in diameter, 5 μm pore size; Macherey Nagel, Germany), a fluorescence detector (excitation and emission wavelengths, 360 and 495 nm, respectively; Waters Model 420, USA) and a computer. The area under the curve in the chromatograms was analyzed using computer software, Borwin Chromatography, Version 1.20 (France).

The mobile phase was a mixture of 250 mM Na acetate buffer (pH: 6.9), distilled deionised water and HPLC grade

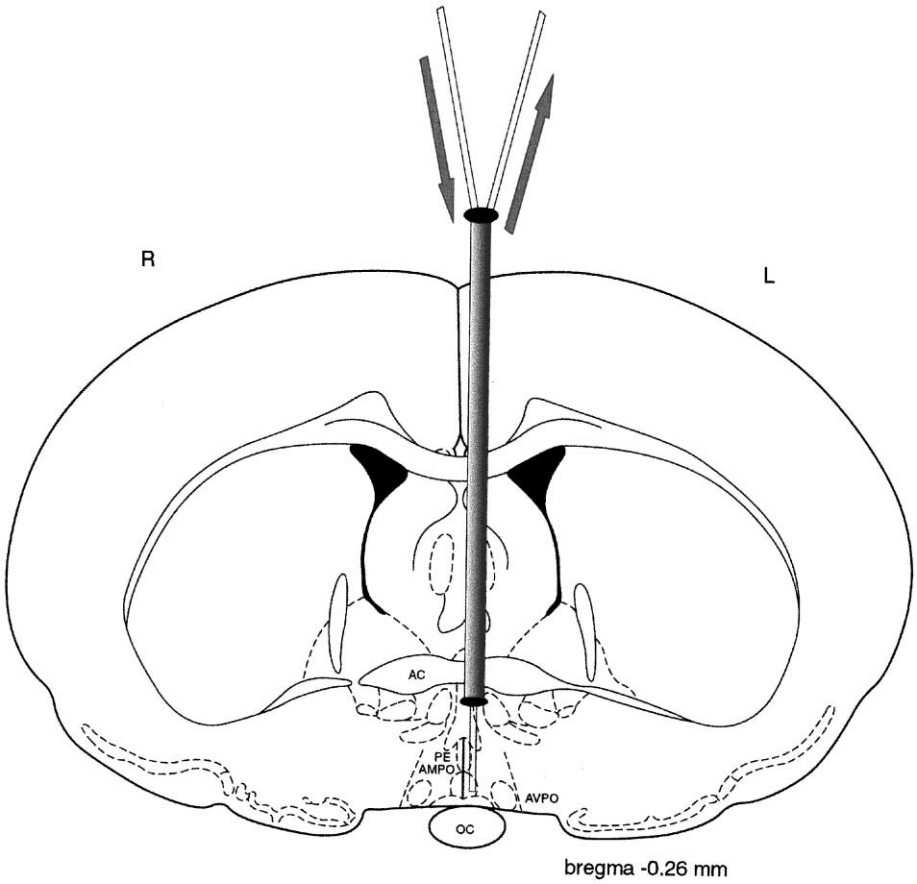


Fig. 1. Schematic representation of anteroventral 3rd ventricle (AV3V) area and proper microdialysis probe placements, adapted from Paxinos and Watson (1986). AC: anterior commissure; AMPO: anteromedial preoptic nucleus; AVPO: anteroventral preoptic nucleus; PE: periventricular hypothalamic nucleus; OC: optic chiasm; L: left side of the rat; R: right side of the rat.

methanol (Labscan, Ireland) at a ratio of 2:1:1 (v/v/v). L-Citrulline (Sigma, USA) was dissolved in 0.1 M HCl in a plastic beaker and aliquots were stored at −20 °C and fresh dilutions of the external standard were prepared daily. Precolumn derivatization was performed by mixing 10 μl of either external standard or the samples with 3 μl derivatizing reagent. The mixture was allowed to derivatize for 2 min in the dark at room temperature. The working derivatization reagent was a mixture of 3-mercaptropropionic acid (in a dilution of 1/10 in methanol) in 1 mg/ml *O*-phytaldialdehyde solution (Sigma) at a final ratio of 1:40 (v/v). Twelve microliters of the samples or standards was injected into the system and the flow rate of the pump was set to 0.60 ml/min.

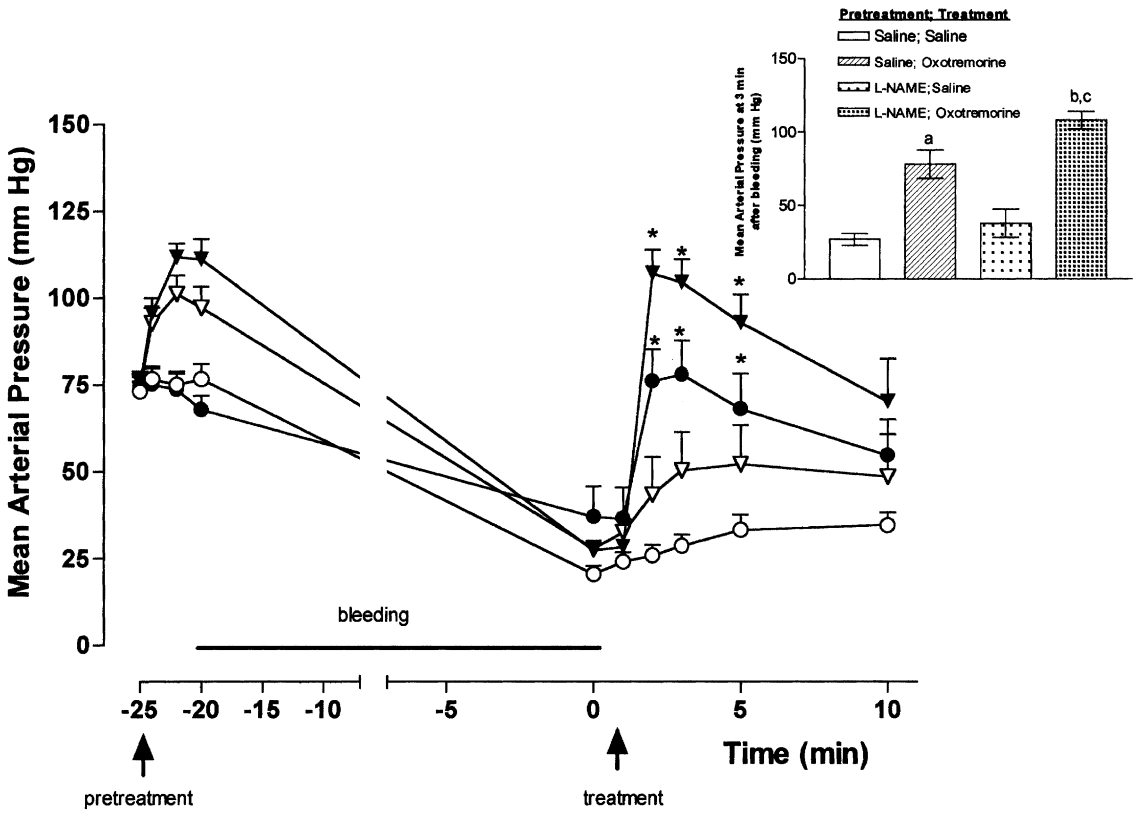
2.6. Experimental protocol

Following microdialysis probe, venous and arterial cannula placement, blood pressure was monitored for 50–60 min and basal dialysates were collected. Then, the rats were bled through left iliac artery for 20 min in three successive steps until the mean arterial pressure fell to and stabilized at approximately 20 mm Hg. A total of 1.99 ± 0.1 ml blood/100 g was withdrawn that is approximately equivalent to 50% of the total blood volume in rats (Collins et al., 1969).

Either physiological saline (0.1 ml/100 g; i.v.) or L-NAME (2.5 mg/kg; i.v.) was injected 5 min prior to the bleeding. After the stabilization of mean arterial pressure at around 20 mm Hg, physiological saline (0.1 ml/100 g, i.v.) or oxotremorine (25 μg/kg; i.v.) was administered. Oxotremorine was given 1 min after the intraperitoneal injection of methylatropine bromide (2 mg/kg) so that peripheral effects of oxotremorine would be prevented. The blood pressure was monitored continuously and if the rats survived for more than 1 h, they were killed by cervical dislocation. Dialysate samples from AV3V probes were collected throughout the experiment as described above.

2.7. Drugs

N^G-nitro-L-arginine methylester (L-NAME), methylatropine bromide, oxotremorine and urethane were pur-



Number of rats surviving

Pretreatment; Treatment \ Time (min)	0	1	3	5	10	15	20	30	40	50	60
○ Saline; Saline (n=12)	12	12	11	10	9	8	7	6	4	4	4
● Saline; Oxotremorine (n=12)	12	12	11	10	9	9	8	8	7	6	6
▽ L-NAME; Saline (n=9)	9	8	7	7	7	7	6	4	4	3	2
▼ L-NAME; Oxotremorine (n=12)	12	12	11	11	10	8	8	8	8	8	6

Fig. 2. The effects of oxotremorine on mean arterial pressure in saline and L-NAME (2.5 mg/kg, i.v.)-pretreated rats subjected to hemorrhagic shock. Data are expressed as means ± S.E.M. The number of rats surviving at different time points are shown in the table. The inset figure shows the means of mean arterial pressure values of different groups at 3 min after bleeding (2 min after the treatment). $^{*}P < 0.05$ vs. time 0; $^{a}P < 0.01$ vs. saline-pretreated, saline-treated group; $^{b}P < 0.01$ vs. L-NAME-pretreated, saline-treated group; $^{c}P < 0.05$ vs. saline-pretreated, oxotremorine-treated group.

chased from Sigma and heparin sodium (Liquemine) was a gift from Roche (Turkey). All drugs were dissolved and/or diluted in saline.

2.8. Data analysis

The results were expressed as "means ± S.E.M.". Mean arterial pressure was calculated as "1/3 pulse pressure + diastolic blood pressure". Analysis of variance for repeated measures (two-way) and post hoc Dunnett's test were used to analyze the effect of oxotremorine on mean arterial blood pressure, by comparing the data points with that obtained at time zero (i.e. just after completion of bleeding; see Fig. 2). One-way analysis of variance and Tukey's post hoc test were used for comparison of the maximum pressor effects in different groups (i.e. 3 min after the completion of bleeding, see Fig. 2). The L-citrulline concentration in saline and in L-NAME pretreated rats was compared using the Kruskal–Wallis test and the 60-min-survival rates were compared using the X^2 test. The level of statistical significance was accepted as $P < 0.05$.

Table 1
The ratios of L-citrulline concentrations (μM) of consecutive 20-min samples collected from the AV3V region after bleeding to the basal values

Pre-treatment; treatment	Time after bleeding (min)		
	0–20	20–40	40–60
Saline; saline	0.7 ± 0.1	1.3 ± 0.5	0.8 ± 0.1
Saline; oxotremorine	1.6 ± 0.8	1.5 ± 0.2	0.9 ± 0.1
L-NAME; saline	1.9 ± 0.8	0.9 ± 0.4	2.7 ± 1.6
L-NAME; oxotremorine	1.1 ± 0.4	0.4 ± 0.1	0.2 ± 0.01

Values are expressed as means ± S.E.M.

3. Results

3.1. The effect of oxotremorine on mean arterial pressure and 60-min survival rate

The basal mean arterial pressure values were not significantly different from each other between groups. The mean arterial pressure of the control group treated with saline fell from 73 ± 4 to 20 ± 2 mm Hg after bleeding (Fig. 2). Only 4 of 12 rats survived 60 min. L-NAME (2.5 mg/kg) given to normotensive rats increased the mean arterial pressure from 74 ± 5 to 97 ± 6 mm Hg within the first 5 min. After bleeding, the mean arterial pressure stabilized at 27 ± 5 mm Hg (Fig. 2). Physiological saline treatment did not restore the mean arterial pressure and survival in this group. Only 2 of 9 rats survived 60 min.

In the saline-pretreated group, oxotremorine at a dose of 25 μg/kg injected after bleeding significantly elevated the mean arterial pressure from 29 ± 3 to 76 ± 9 mm Hg in 2 min ($P < 0.01$; Fig. 2). Oxotremorine at this dose did not improve survival significantly and only 6 of 12 rats survived 60 min.

L-NAME pretreatment did not change the effects of the saline treatment but oxotremorine induced a higher pressor effect in L-NAME-pretreated hypovolemic rats by increasing the mean arterial pressure values from 27 ± 2 to 107 ± 7 mm Hg in 2 min. When the mean arterial pressure values at time 3 min were compared by one-way analysis of variance, the oxotremorine-induced pressor effect was significantly higher in L-NAME-pretreated rats ($P < 0.05$) (see inset in Fig. 2). However, the 60-min survival rate in the L-NAME-pretreated oxotremorine group was not significantly different from that of the saline-pretreated group (6 of 12 rats survived 60 min).

3.2. L-Citrulline levels in AV3V region

No statistically significant difference between groups was observed for the basal L-citrulline levels (Table 1). The mean basal L-citrulline concentration of all rats was 1.99 ± 0.3 μM ($n = 18$). Bleeding to hypovolemic shock increased the L-citrulline concentration up to a mean of 3.22 ± 0.9 μM in saline-pretreated rats ($n = 10$), where the concentration of L-citrulline was found to be significantly lower (1.0 ± 0.4 μM) in L-NAME-pretreated rats ($n = 8$) ($P < 0.05$; Fig. 3). Neither physiological saline nor oxotremorine treatments affected L-citrulline concentrations further in either saline- or L-NAME-pretreated rats (Table 1).

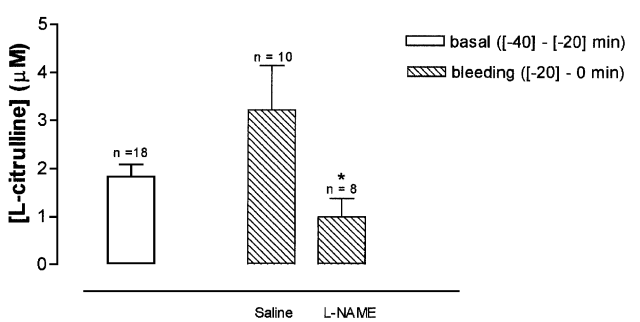


Fig. 3. The concentrations of L-citrulline in AV3V region before (basal) and during bleeding in saline-and L-NAME-pretreated rats. * $P < 0.05$ vs. saline-pretreated rats.

4. Discussion

Cholinomimetic agents were shown to produce pressor responses via central muscarinic receptors (Aslan et al., 1997; Brezenoff and Giuliano, 1982; Brezenoff and Rusin, 1974). Furthermore, these agents also produce a rapid improvement of the hypotension and increase survival rate in experimental hemorrhagic shock models through central cholinergic actions (Bertolini et al., 1989; Guarini et al., 1989). We had previously reported that i.v. oxotremorine at a dose of 50 μg/kg significantly increased blood pressure and 60 min survival rate in rats subjected to hemorrhagic shock (Onat et al., 1994). Muscarinic receptors have been shown to be involved in the pressor effect, whereas nicotinic receptors mediate the decrease in mortality, by using the muscarinic antagonist atropine (2 mg/kg, i.v.), and the nicotinic antagonist mecamylamine (60 μg/rat, i.c.v.) (Guarini et al., 1989; Onat et al., 1994).

The present study provided evidence that NOS inhibition by L-NAME potentiates the pressor effect of the cholinomimetic agent, oxotremorine, in rats in hemorrhagic shock. NO plays an important role in the regulation of blood pressure and regional blood flow (Das and Kumar, 1995; Ignarro et al., 1987; Moncada et al., 1991; Rees et al., 1989). The acute administration of NOS inhibitors causes an increase in blood pressure, as shown in the present study, which is associated with a decrease in NO production (Klabunde et al., 1991; Rees et al., 1989; Zatz and Nucci, 1991). The chronic administration of NOS inhibitors also produces sustained hypertension (Baylis et al., 1992; Riberio et al., 1992). The sympathetic nervous system and renin–angiotensin–aldosterone system were suggested to contribute to these NO-mediated effects (Jover et al., 1993; Liu et al. 1998; Melaragno and Fink, 1996; Pollock et al., 1993).

Endotoxic and hemorrhagic shock are conditions where abnormal NO production was postulated to play a key pathogenic role (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990; Thiemermann et al., 1993; Wright et al., 1992; Zingarelli et al., 1992). Besides causing vascular hyporeactivity to norepinephrine, NO also inhibits norepinephrine release from sympathetic nerve terminals (Schwartz et al., 1995; Thiemermann et al., 1993). ACTH and its fragments were shown to exert antishock effects in rats in hemorrhagic shock via central muscarinic receptors

(Bertolini et al., 1986, 1989; Guarini et al., 1990). Increased blood NO levels during hemorrhagic shock have been reported and ACTH-induced reversal of the shock state was, in turn, claimed to be associated with the normalization of blood NO levels (Bazzani et al., 1997). The same group has demonstrated that inhibition of NOS both by a non-selective inhibitor, L-NAME, and a selective inducible NOS inhibitor, *S*-methylisothurea, potentiated the cardiovascular and respiratory effects of ACTH in the rat hemorrhagic shock model (Bazzani et al., 1989). This potentiation was reversed by L-arginine, supporting the conclusion that NO overproduction contributes to the hypotension in hypovolemic shock state.

It seems likely that ACTH and cholinomimetic agents share some central cholinergic pathways in their antishock effects. Therefore, in the present study, it was planned to test the involvement of NO in the effect of oxotremorine on blood pressure and survival of rats bled to shock. Oxotremorine was used at half the dose that had been shown to raise mean arterial pressure about 100 mm Hg and increased 60-min survival rate by 92% (Onat et al., 1994). At this dose (25 μg/kg, i.v.), it produced a smaller, but significant pressor effect; however, survival could not be improved. In L-NAME-pretreated rats, the oxotremorine-induced blood pressure increase was significantly higher. However, NOS inhibition in addition to oxotremorine treatment failed to potentiate the effect on the mortality rate. The reason for this discrepancy in the effects of L-NAME on blood pressure and survival might be the low dose of oxotremorine used, so that L-NAME-induced potentiation could not reach a significant level. Another possible explanation might concern the cholinergic receptor. Since it was reported that oxotremorine restores blood pressure via central muscarinic receptors and improves survival via nicotinic receptors in rats subjected to hemorrhagic shock (Onat et al., 1994), whether L-NAME potentiates muscarinic actions but not nicotinic actions of oxotremorine needs to be further investigated. In conclusion, the present study provided further evidence for the role of NO in hemorrhagic shock and demonstrated that the overproduction of NO counteracts the beneficial effect of oxotremorine as shown previously in the case of ACTH.

Since the cholinergic pressor effect is indeed a combination of responses originating from several brain regions, we decided to investigate any potential change in NO production in a selected brain structure, the AV3V region, which was previously shown to be actively involved in oxotremorine-induced reversal of hypotension in the hemorrhagic shock model in rats (Onat et al., 1994). NO is a very labile biomolecule and its production is difficult to detect under many experimental conditions. On the other hand, L-citrulline is known to be produced in equal amounts during NO synthesis from L-arginine (Bredt and Snyder, 1994). It was also known that availability of arginine is one of the rate-limiting factors in cellular NO production. Citrulline, which is formed as a by-product of the NOS reaction, can be recycled to arginine by successive actions of argininosuccinate synthase and argininosuccinate lyase, forming the citrulline–NO cycle in neuronal PC12 cells (Mori and Gotoh, 2000). Additionally, it was shown that L-citrulline immunocytochemistry is a useful technique to detect NOS activity in brain (Keilhoff et al., 2000). Therefore, extracellular L-citrulline levels of the microdialysis samples collected via microdialysis probes inserted into the AV3V region were determined to provide indirect information about NO generation.

Bleeding to shock increased L-citrulline concentration in the AV3V region significantly in control rats and L-NAME pretreatment abolished this increase. These findings indicate that NO production in this area contributes to the hypotension due to hemorrhage. Although L-NAME potentiated the pressor effect of oxotremorine, indicating an interaction between central cholinergic system and NO synthesis, this cholinomimetic agent did not produce any significant difference in L-citrulline levels in AV3V. It may be concluded that cholinergic–nitrergic interaction can occur at several other brain structures.

Collectively, the results of this study reveal that NO production contributes to hypotension in rats bled to shock since NO levels in the AV3V region increase in response to bleeding and since this increase is abolished and the oxotremorine-induced reversal of hypotension is potentiated by NOS inhibition.

Acknowledgements

The authors wish to thank to Dr. Nural Bekiroglu from the Department of Biostatistics (Marmara University School of Medicine, Istanbul, Turkey) for her valuable comments on data analysis. This study was supported by "NOVARTIS Pharmacology-1998 Grant, Turkey".

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